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Use of smear bacteria and yeasts to modify flavour and appearance of Cheddar cheese

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ABSTRACT

The strains *Staphylococcus saprophyticus* DPC5671 and *Corynebacterium casei* DPC5298 were applied in combination with *Debaryomyces hansenii* DPC6258 to the surface of young Cheddar cheese curd to obtain two different smear-ripened cheeses. A surface microbiota developed over the incubation period, comprising of both yeast and bacteria; pulsed field gel electrophoresis confirmed that the inoculated strains of *S. saprophyticus* DPC5671 or *C. casei* DPC5298 were the dominant bacterial strains on the surface of the cheese at the end of the ripening period. The smear cultures changed the appearance and aroma, which were significantly different from the control cheese. The approach presented in this study represents a method for the development of new cheese varieties with novel aromas within a short ripening time.

1. Introduction

Smear cheese is a traditional dairy product, which plays an important role in both small and industrial scale dairy production. Smear cheese is characterised by a short ripening time and strong aroma produced by the growth of smear microbiota on the cheese surface. Smear-ripened cheeses are manufactured by inoculating the surface of the cheese curd, dipping, spraying or brushing with a mixture of bacteria and yeasts. The traditional method of production is called "old-young smearing" and consists of washing young curds with the brine from old cheese, to encourage the transfer of the microbiota from the old to the young cheeses (Desmasures, Bora, & Ward, 2015; Fox, Guinee, Cogan, & McSweeney, 2017a).

The microbiota on the surface of the smear cheese is composed of a variety of microorganisms that coexist in symbiotic relationships. Yeasts are normally the first resident microorganisms to establish themselves on the surface of the cheese due to their tolerance to low pH and salt. Yeasts metabolise lactate, producing H_2O and CO_2 and increase the pH (Cholet, Hénaut, Casaregola, & Bonnarme, 2007; Corsetti, Rossi, & Gobbetti, 2001). Moreover, they produce metabolites and growth factors (vitamins and amino acids) which encourage the growth of Gram-positive bacteria, such as *Corynebacterium*, *Staphylococcus* and *Brevibacterium* species (Cogan et al., 2014; Larpin et al., 2011).

The growth of smear microorganisms on the surface of cheese curd modifies the appearance, aroma, proteolysis and lipolysis of the cheese within a relatively short ripening time (McSweeney, 2004). The combined growth of the bacteria and yeasts on the surface of the cheese results in the production of proteolytic and lipolytic enzymes, increasing the amount of free amino acids (FAAs) and free fatty acids (FFAs) (McSweeney & Sousa, 2000; Sousa, Ardö, & McSweeney, 2001). Yeasts and Gram-positive bacteria isolated from smear cheeses have a wide range of proteolytic enzymes that display various peptidase activities,

with FAAs increasing within the cheese as a consequence. Additionally, yeasts and Gram-positive bacteria possess esterolytic/lipolytic enzymes capable of catabolising triacylglycerols in cheese, producing FFAs (Curtin, Gobbetti, & McSweeney, 2002; Fox, Guinee, Cogan, & McSweeney, 2017b).

The further metabolism of FAAs and FFAs during the ripening produces flavour compounds important for cheese aroma. The catabolism of FAAs, especially of branched chain amino acids, aromatic amino acids and sulphur amino acids, produces mainly aldehydes, alcohols, carboxylic acids and sulphur compounds. Moreover, FFAs are involved in reactions leading to the production of flavour compounds such as secondary alcohols, carboxylic acids, esters, lactones and ketones (McSweeney & Sousa, 2000; Singh, Drake, & Cadwallader, 2003; Smit, Smit, & Engels, 2005; Yvon & Rijnen, 2001).

The characteristics of smear-ripened cheese are not strictly controlled inside artisanal smear cheese plants. The resulting product is also affected by the final microbial consortia of the cheese, which is influenced by the individual in-house microbiota of the cheese-making facilities. Microorganisms detected in the environment of artisanal cheese-making plants have also been found on the surface of smear cheeses, indicating a strong relationship between product and the environment in which the cheese is manufactured and ripened (Bokulich & Mills, 2013; Goerges et al., 2008; Mounier et al., 2006a).

In previous studies, smear strains were added to the cheese surface or as adjunct cultures to the milk during manufacture of smear-ripened cheese; however, some of the added strains were not detected at the end of ripening (Feurer, Vallaeys, Corrieu, & Irlinger, 2004; Goerges et al., 2008). These commercial smear strains have to compete with the in-house microbiota and do not always successfully establish themselves on the cheese surface (Bokulich & Mills, 2013; Feurer et al., 2004; Goerges et al., 2008). It is likely that the

relationship within the smear microbiota promotes the survival of a particular group of microorganisms to the detriment of others.

With the abolition of the milk quotas within the EU in 2015 there is a renewed interest in developing novel cheeses with a range of flavours. There is a progressive increase in global cheese consumption, with an annual production in Ireland of 207,100 tonnes in 2015 (data from Eurostat). Therefore the aim of this work was to develop a novel cheese with diverse aromas and short ripening time using cheese curd made in a traditional Cheddar cheese plant. Ripening time for Cheddar cheese can be from a little as 3 months for mild cheese up to > 9–12 months for mature/extra mature varieties. In this study, the ability of smear bacteria and yeast to grow on the surface of young Cheddar cheese curd was investigated to produce a cheese variety with different flavour and appearance compared with Cheddar cheese within a short time frame of 35 days.

2. Materials and methods

2.1. Preparation of smear suspensions

For the preparation of *Debaryomyces hansenii* DPC6258 suspension, the strain was streaked onto yeast extract glucose chloramphenicol agar (YGC agar; Becton, Dickinson and Company, City West, Dublin, Ireland) and incubated aerobically at 25 °C for 96 h. Using a 5 µL loop, the strain was inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company) and incubated, shaking at 100 rpm, at 25 °C. When the OD_{600} reached ~1, the cells were centrifuged at 6000 × *g* at 4 °C for 15 min, washed twice with sterile 0.75% NaCl and the pellet was resuspended in sterile 0.75% NaCl to obtain a suspension of ~10^6 cfu mL^{-1}.

For the preparation of the *Corynebacterium casei* DPC5298 and *Staphylococcus saprophyticus* DPC5671 suspensions, the strains were streaked onto trypticase soy agar (TSA; Becton, Dickinson and Company) and incubated aerobically at 30 °C for 48 h. Using a 5 µL loop, the strains were inoculated into 10 mL of trypticase soy broth (TSB; Becton, Dickinson and Company) and incubated, shaking at 100 rpm, at 30 °C. When the OD_{600} reached ~1, the cells were centrifuged at 6000 × *g* at 4 °C for 15 min, washed twice with sterile 0.75% NaCl and the pellets resuspended in sterile 0.75% NaCl to obtain a suspension of ~10^5 cfu mL^{-1}.

2.2. Smearing of cheese blocks

Cheddar cheese was supplied by a commercial cheese company as 20 kg blocks, <24 h post manufacture. The large cheese block was aseptically cut into smaller blocks (~8 × 6.5 × 30 cm). These blocks were then inoculated by placing them in a saline suspension containing *D. hansenii* DPC6258 (10^6 cfu mL^{-1}), ensuring an even coating of yeast. Subsequently the blocks of cheese were placed on sterile, plastic coated racks and allowed to drain. Once the excess liquid had completely drained, the cheese pieces were placed inside a sterile plastic bag (Südpack Verpackungen, Ochsenhausen, Germany) on the rack ensuring that the sides of the cheese did not make contact with the plastic bag. Relative humidity % (RH%) was maintained by pouring 100 mL of sterile 0.75% NaCl into the base of the bag and the bag was sealed. The cheese was ripened at 15 °C with a RH% of ~97%. After 5 days of ripening, the blocks of cheese were removed from the bag and dipped in saline suspension containing *C. casei* DPC5298, or *S. saprophyticus* DPC5671 (10^5 cfu mL^{-1}). The blocks of cheese were placed on the sterile rack and incubated for a further 30 days (for a total ripening period of 35 days), as described above, to produce a smear cheese with *D. hansenii* DPC6258

in combination with *S. saprophyticus* DPC5671 (cheese A) or *C. casei* DPC5298 (cheese B). During the ripening period the surface of the cheese blocks was washed with a sterile sponge soaked in a sterile brine solution (5% NaCl) at day 7, 10, 15 of ripening to ensure an even growth of the smear microbiota. As a control, blocks of Cheddar cheese were vacuum packed in sterile bags and incubated at 15 °C. These blocks were not smeared with either bacteria or yeasts and were not washed with NaCl solution during the ripening period. However, the control cheese differs from normal Cheddar cheese in that the temperature of ripening was higher (15 °C) than the ripening temperature normally associated with Cheddar cheese (~8 °C). Three replicate cheese trials were performed.

2.3. Sampling cheese

The surface of the cheese A and B was aseptically sampled for enumeration and isolation of bacteria and yeast at 3, 5, 7, 10, 15, 21, 25, 30 and 35 days of ripening. When analysing the control cheese samples, a composite sample of core and surface was analysed, while for the test cheeses both the surface and core were analysed separately. At day 0 and day 35, samples were taken from the control cheese, cheese A and B for composition, urea-polyacrylamide gel electrophoresis (urea-PAGE), free fatty acid and free amino acid analysis. At day 35, samples were taken from the control cheese, cheese A and B for sensory evaluation and volatile analysis. At days 0, 10, 15, 21, 25, 30, 35 samples were taken from the control cheese, cheese A and B for proteolysis analysis and colorimetric analysis.

2.4. Enumeration of bacteria and yeast from cheese surface

During ripening, ~ 5 cm^2 of the surface of the test cheeses were aseptically removed and resuspended in 2% trisodium citrate, serially diluted and plated on TSA 5% NaCl with 50 U mL^{-1} of nystatin (Sigma Aldrich, Arklow, Co. Wicklow, Ireland) and YGC agar, for the enumeration of smear bacteria and yeasts, respectively. Nystatin was added to TSA to prevent the growth of yeast and moulds. The TSA plates were incubated at 30 °C for 48 h, while YGC plates were incubated at 25 °C for 96 h. Colonies were counted and the results expressed as log cfu g^{-1} of cheese. Five colonies from the highest countable dilution were re-streaked onto TSA and incubated at 30 °C for 48 h. Isolates were stocked at −80 °C in glycerol for further analysis.

2.5. *Pulsed field gel electrophoresis*

The cultures isolated from cheese A and B were grown on TSA, incubated aerobically at 30 °C for 24 h and then inoculated in 8 mL of TSB, with shaking at 100 rpm at 30 °C for 24 h. Pulsed field gel electrophoresis (PFGE) was carried out as described by Bannerman, Hancock, Tenover, and Miller (1995) for *S. saprophyticus*, while the method outlined by Brennan et al. (2001) was used for *C. casei*. Before digestion the agarose plugs were cut into small slices (1 by 2 mm), transferred into 100 µL restriction buffer containing 20 U of *Sma*I for *S. saprophyticus*, and 20 U of *Spe*I (all from New England Biolabs, Hitchin, UK) for *C. casei* and incubated over night at 25 °C or 37 °C, respectively. The gel was run in a CHEF-DR III PFGE apparatus (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, UK) at 1 V (6 V cm^{-1}) at 14 °C for 20 h, with the pulse ramped from 5 to 40 s for *S. saprophyticus,* while 1 V (6 V cm^{-1}) at 14 °C for 16 h, from 1 to 20 s for *C. casei*. Gels were stained with ethidium bromide (0.5 µg mL^{-1}) in water, destained in water and then photographed using a gel imaging system (AlphaImager 2000, Alpha Immotech, San Leonardo, CA, USA).

2.6. Compositional analysis

Cheese samples were analysed for moisture content by oven-drying 3 g of cheese sample at 102 °C for 5 h and for salt content by a potentiometric method on a mixture of 2 g of grated cheese and 60 g of water (IDF, 1988). Total protein was determined on 0.2 g of cheese sample and total fat was determined on 3 g of cheese sample by standard methods (IDF, 1993, 1996). The pH was measured with a standard pH meter (Mettler-Toledo MP220, Schwerzenbach, Switzerland) on a mixture of 20 g of grated cheese and 12 g of water, as described by British Standards Institution standard (BSI, 1976).

2.7. Determination of colour

The development of the colour during the ripening was measured in triplicate on the surface of the cheese at room temperature, using a Minolta Colorimeter CR-300 (Minolta Camera, Osaka, Japan). A white colour tile standard was used to calibrate the instrument and the colour was analysed using L*, a* and b*-values, which describe the colour space. L*-value measures the visual lightness (as values increase from 0 to 100), a*-value measures from the redness to greenness (positive to negative values, respectively) and b*-value from the yellowness to blueness (positive to negative values, respectively).

2.8. Proteolysis

Proteolysis was determined by measuring the levels of the non-casein nitrogen content, soluble at pH 4.6 (pH4.6-SN) (Fenelon & Guinee, 2000) and total nitrogen (TN) on a

water soluble extract of a mixture of 60 g of grated cheese and 120 g of water, using the macro-Kjeldahl method (IDF, 1993). The levels of proteolysis were expressed as a percentage of the ratio between non-casein nitrogen content and total nitrogen (% pH4.6-SN/TN).

2.9. Urea-polyacrylamide gel electrophoresis

Urea-polyacrylamide gel electrophoresis (urea–PAGE) was performed according to the method described by Rynne, Beresford, Kelly, and Guinee (2004). The gel system was composed of a separating and stacking gel, using a PROTEANs II xi cell vertical slab gel unit (Bio-Rad Laboratories Ltd). The samples were prepared, maintaining the same concentration of protein (4.25 g of protein content L^{-1} sample buffer) to have a final volume of 1 mL. Ten microlitres of sample solution were loaded into individual wells. The sample buffer (pH 8.7), the sample preparation and the running conditions were as described by Henneberry, Wilkinson, Kilcawley, Kelly, and Guinee (2015). After the run, the gel was removed from the plates and stained overnight in an aqueous solution of Coomassie Blue G250 (0.25%, w/v), destained in a destaining solution (acetic acid 10%, methanol 25%), and washed in distilled water. The images were acquired by a gel imaging system (AlphaImager 2000; Alpha Immotech).

2.10. Free amino acid analysis

Individual FAAs were determined on the soluble N extracts as described by McDermott et al. (2016) using a Jeol JLC-500V AA analyser fitted with a Jeol Na+ high performance cation exchange column (Jeol Ltd., Garden City, Herts, UK). The

chromatographic analyses were conducted at pH 2.2. Results are expressed as µg mg^{-1} of cheese.

2.11. Free fatty acid analysis

FFA extraction was performed on 10 g of grated cheese, according to the method described by De Jong and Badings (1990). The FFA extracts were aliquoted into amber glass vials and capped with PTFE/white silicone septa (Agilent Technologies, Little Island, Cork, Ireland). The FFA extracts were derivatised as methyl esters as outlined by Mannion, Furey, and Kilcawley (2016) using a Sample Prep Workbench (Agilent Technologies). Fatty acid methyl esters extracts were analysed using Varian CP3800 gas chromatograph (Aquilant, Dublin, Ireland) with a CP84000 auto-sampler and flame ionisation detector (GC-FID) and a Varian 1079 injector (Aquilant). For the GC-FID analysis, 0.7 µL were injected into a CP FFAP CB capillary column (30 m × 0.25 mm × 0.32 µm) (Agilent Technologies). Results are expressed as µg mg^{-1} of cheese.

2.12. Volatiles analysis

After sampling the cheese samples were wrapped in foil, vacuum packed and stored at –20 °C. Before analysis, the samples were defrosted at room temperature and blended with a cheese grater. For the analysis, 4 g of cheese sample were placed in a screw capped SPME vial with a silicone/PTFE septum (Apex Scientific, Maynooth, Ireland). The SPME vials were equilibrated to 40 °C for 10 min with pulsed agitation (5 s on, 2 s off) at 500 rpm. Sample introduction was performed using AOC-5000 injection system (Shimadzu, Albert-Hahn-Str., Duisburg, Germany) and a single 50/30 µm Carboxen TM137 / divinylbenzene /

polydimethylsiloxane (DVB/CAR/PDMS) SPME fibre was used for the volatile extraction (Agilent Technologies). The SPME fibre was exposed to the headspace above the samples for 20 min at depth of 54 mm, then was retracted and injected into the GC inlet and desorbed for 2 min at 250 °C. Injections were made on a Shimadzu 2010plus with an Agilent DB-5ms column (60 m × 0.25 mm × 0.25 µm) (Agilent Technologies), using a multipurpose injector with a Merlin microseal. The temperature of the column oven was initially held for 30 s at 35 °C, increased to 230 °C at a rate of 6.5 °C min^{-1}, and to 320 °C at a rate of 15 °C min^{-1}, yielding at total GC run time of 41.5 min. The carrier gas, helium, was at a constant pressure of 1.58 bar, performing a splitless injection. The detector, a Shimadzu TQ8030 MSD triple quadrupole mass spectrometer, was used in single quadrupole mode. The ion source temperature and interface temperature were set at 220 °C and 280 °C, respectively, and the MS mode was electronic ionisation (70 v) with the mass range between 35 and 250 amu. The chromatograms were deconvoluted and the peaks quantified with TargetView (Markes International Ltd, Llantrisant, UK). The compounds were identified using mass spectra comparisons to the NIST 2014 mass spectral library, and using an internal database with known target and qualifier ions for each compound. Ten microlitres of the standard solution [1-butanol, dimethyldisulphide, butyl acetate, cyclohexane, benzaldehyde at 10 ppm, and 2-phenylethanol at 50 ppm, in methanol/:water (1:99)] in a SPME vial were run before and after every series of samples to ensure that both the SPME extraction and MS detection were performing within specification. An autotune of the GCMS was carried out prior to the analysis to ensure optimal GCMS performance. All analyses were performed in triplicate.

2.13. Sensory affective evaluation and ranking descriptive analysis

The blocks of cheese sample (~500 g), vacuum packed and stored at -20 °C, were defrosted at 4 °C the day before sensory evaluation. The surface of cheese A and B was removed and the blocks were cut into portions for the sensory test. Twenty naive assessors were recruited in University College Cork, Ireland. Sensory acceptance testing was conducted using these untrained assessors, who were both Cheddar and smear cheese consumers, (age 21–48 years). Assessors used the sensory hedonic descriptors for the control, cheese A and B. Samples underwent monadic presentation to the panel at ambient temperatures (~21 °C) and coded with a randomly selected 3 digit code. Each assessor was asked to indicate their degree of liking on a 10 cm line scale ranging from 0 (extremely dislike) to 10 (extremely like). Ranking descriptive analysis (RDA) was then undertaken using the consensus list of sensory descriptors, which was also measured on a 10 cm line scale. All samples were presented in duplicate.

2.14. Statistical analysis

The statistical analysis for cheese composition, sensorial analysis, FAAs and FFAs were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab Inc., Coventry, UK). A split plot designed with SAS 9.3 (SAS, Dublin, Ireland) was used to determine the singular effect of smear treatments, ripening time and their interaction on the microbiology, pH, % pH 4.6-SN/TN, L*, a* and b* -values, measured at several time points during the ripening. The statistical analysis of the volatile compounds was tested with ANOVA-Partial Least Squares Regression (APLSR) using Unscrambler (The Unscrambler X 10.3, Camo Software, Oslo, Norway). From the results of the APLSR, the individual volatile compounds positively correlated with the samples were tested with one-way analysis of variance (ANOVA) using Minitab 17 (Minitab Inc.), to evaluate the significant differences

among the samples for each volatile compound. The level of significance for all analyses was determined at $P < 0.05$.

3. Results

3.1. Growth of the strains and pH development

PFGE analysis established that the inoculated cultures of *S. saprophyticus* DPC5671 and *C. casei* DPC5298 were the dominant bacterial strains isolated at the end of ripening (day 35) (supplementary data). The total count of yeasts and smear bacteria during ripening is shown in the Fig. 1. A significant interactive effect ($P < 0.05$) between ripening time and smear treatments was observed for the growth of the surface microbiota. No significant differences were observed on the growth of yeast and bacteria between cheese A and cheese B.

The variation in pH of the control, and the core and the surface of cheese A and B is shown in Fig. 2. A significant interactive effect ($P < 0.05$) between smear treatments and ripening time was observed for pH. From day 21 to 35, the pH was significantly higher ($P < 0.05$) at the surface of cheese A compared with its respective core and the control. From day 15 to 35, the pH was significantly higher ($P < 0.05$) at the surface of cheese B compared with the respective core and the control.

3.2. Cheese composition

The mean composition of the Cheddar cheese before the smearing process was typical of a commercial Cheddar cheese after manufacture, but the smearing treatments influenced

the compositional parameters at the end of the ripening (35 days). Compositional data and significant differences (P <0.05) are presented in Table 1.

3.3. Proteolysis

The proteolysis was particularly high at the surface of cheese A and B, where the smear treatments significantly (P <0.05) influenced the level of % pH 4.6-SN/TN (supplementary data). Urea-PAGE (Fig. 3) confirmed the high level of proteolysis on the surface of cheese A and B. α_{S1}-Casein was partially degraded to α_{S1}-CN (f102–199) and α_{S1}-CN (f24–199) in the control cheese and in the core of cheese A and B. However there was evidence of further breakdown products on the surface samples of the test cheeses. Similar β-casein degradation patterns were observed for the control and core of test cheeses, while samples from the surface of the test cheeses showed that the β-casein was almost totally degraded producing β-CN (106–209) and β-CN (108–209) with higher intensity (Fig. 3).

3.4. Free amino acids and free fatty acids

Significant differences (P <0.05) on the total amount of FAAs were observed between the control and the surface of cheese A and B at the end of ripening on day 35 (Fig. 4). In the cheese A and B, some individual FAAs were significantly higher (P <0.05) on the surface compared with their respective cores or the control. No significant difference was determined between the surface samples of cheese A and B with respect to the total FAA content.

Significant differences (P <0.05) in the total amount of FFAs were observed between the control and the surface of cheese A and B at day 35 (Fig. 5). In the cheese A and B, all individual FFAs detected on the surface were significantly higher (P <0.05) compared with

362 the levels of the respective cores or the control. Significantly higher levels (P <0.05) of total
363 FFAs were detected on the surface of cheese A (20169±2120 µg mg^{-1}) compared with the
364 surface of cheese B (12338±3382 µg mg^{-1}).
365
366 *3.5. Cheese colour*
367
368 The measurements of the colour on the surface of cheese A and B during ripening are
369 shown in Fig. 6. A significant interactive effect (P <0.05) between time and smear treatments
370 was observed on a* and b* values. From day 15, a* value was significantly higher (P <0.05)
371 compared with the control for the cheese A and B. At the end of the ripening (day 35), a*
372 value was significantly higher (P <0.05) for cheese B compared with cheese A, resulting a
373 redder colour on the surface of cheese B.
374
375 *3.6. Volatile compounds and sensory analysis*
376
377 The analysis of variance enabled the selection of 40 volatile compounds that were
378 significantly different (P <0.05) and positively correlated with the samples (Table 2). In total,
379 22 volatile compounds (7 acids, 4 alcohols, 5 esters, 4 sulphur compounds, 1 ketone and 1
380 aromatic hydrocarbon) were significantly associated (P <0.05) with the surface of cheese A.
381 Eight compounds (2 acids, 2 aldehydes, 1 alcohol 1 ester, 1 pyrazine and 1 sulphur
382 compound) were significantly associated with the surface of cheese B. Two compounds (1
383 ketone and 1 hydrocarbon) were significantly associated (P <0.05) with the core of cheese A.
384 Two compounds (1 alcohol and 1 ketone) were significantly associated (P <0.05) with the
385 core of cheese B. Nine compounds (4 alcohols, 4 ketones and 1 ester) were significantly
386 associated (P <0.05) with the control cheese.

Using hedonic sensory analysis (Fig. 7) the control cheese scored significantly higher (P <0.05) for "Liking of Aroma" compared with the cheese A and B. Cheese A scored significantly higher (P <0.05) for "Liking of Aroma" compared with cheese B. The control and cheese A scored significantly higher (P <0.05) for "Liking of Flavour" compared with cheese B. As seen in Fig. 8, the control cheese, as expected, scored significantly higher (P <0.05) for "Cheddar flavour" compared with cheese A, which scored significantly higher (P <0.05) for "Cheddar flavour" compared with cheese B. Cheese A and B scored significantly higher (P <0.05) for "Pungent flavour" and "Mould cheese flavour" compared with the control cheese. Cheese B scored significantly higher (P <0.05) for descriptors "Sweaty/Sour Aroma", "Pungent Aroma" and "Off-Aroma" compared with the control cheese. The control scored significantly higher (P <0.05) for "Crumbly texture" compared with cheese A and B.

4. Discussion

C. casei and *S. saprophyticus*, bacteria commonly isolated from smear-ripened cheeses (e.g., Limburger, Reblochon, Livarot, Tilsit, Gubbeen) (Cogan et al., 2014; Larpin et al., 2011), do not belong to the traditional microbiota of Cheddar cheese, although in this study both strains established themselves on the surface of young Cheddar cheese curd and they were the dominant population on the cheese surface throughout the ripening.

C. casei DPC5298 or *S. saprophyticus* DPC5671 in combination with *D. hansenii* DPC6258 developed a coloured layer on the cheese surface after 15 days of ripening. Unlike studies with *Brevibacterium linens* that showed that colour development was influenced by the yeast strain used (Leclercq-Perlat, Corrieu, & Spinnler, 2004a), in this study the type of colour developed was dependent on the bacteria used. The combination *D. hansenii* DPC6258 with *C. casei* DPC5298 on cheese B developed a redder colour compared with the

412 combination of *D. hansenii* DPC6258 with *S. saprophyticus* DPC5671 on cheese A. Similar
413 results were shown by Mounier et al. (2006b) who reported in a cheese model an increase of
414 the colour after the 15th day of ripening at 14 °C and higher a* value for the combination *D.*
415 *hansenii* with *C. casei*, compared with *D. hansenii* combined with *S. saprophyticus*, resulting
416 in higher development of red colour.

417 The development of the typical aroma and flavour in cheese A and B is associated
418 with the lipolytic and proteolytic processes of the yeast and bacterial component of the smear
419 consortium. These processes were slower in the control cheese, which did not develop the
420 same levels of proteolysis, FAAs and FFAs in the short ripening time (35 days).

421 During cheese ripening, a gradual decomposition of caseins into small peptides and
422 FAAs occurs. FAAs are considered precursors of flavour compounds during the development
423 of cheese flavour. It is likely that the smear treatments increased the proteolysis of β-casein
424 by plasmin (more active at alkaline pH) on the surface of the test cheeses (Fig. 3), increasing
425 the pH levels. At the end of ripening the dominant FAAs detected in high amount on the
426 surface of the test cheeses were valine, leucine, proline, glutamate and lysine (Fig. 4), due to
427 their relative concentration in casein and the peptidase activity of the smear consortium,
428 especially *D. hansenii* DPC6258. Analysis of cell free supernatants of the cultures used in
429 this study confirmed that *D. hansenii* DPC6258 had high peptidase activities (PepX and
430 PepN), while activities were low for *S. saprophyticus* DPC5671 and *C. casei* DPC5298
431 (supplementary data). Similar results have been previously reported for other *D. hansenii*, *S.*
432 *saprophyticus* and *C. casei* strains (Bintsis, Vafopoulou-Mastrojiannaki, Litopoulou-
433 Tzanetaki, & Robinson, 2013; Casaburi, Villani, Toldrá, & Sanz, 2006; Curtin et al., 2002).

434 The hydrolysis of triglycerides is the main biochemical transformation of fat during
435 cheese ripening, which leads to the production of FFAs. Individual FFAs contribute to the
436 cheese aroma with their specific flavours and especially with their metabolites. At the end of

ripening high amounts of FFAs were detected on the surface of cheese A and B. The levels of all individual FFAs detected were higher on the surface of cheese A compared with cheese B, especially for C10:0 (Fig. 5). It has been previously reported that the lipolysis by *D. hansenii* is weak, while studies on Gram-positive bacteria showed good activity on substrates with different glyceride chains length (Bintsis et al., 2003; Cardoso et al., 2015; van den Tempel & Jakobsen, 2000). Experimental work showed that *S. saprophyticus* DPC5671 had greater lipolytic activity on tributyrin than either *C. casei* DPC5298 or *D. hansenii* DPC6258 (supplementary data). These results are in agreement with what was previously reported by Talon and Montel (1997), who detected lipolytic activity on tributyrin in a range of staphylococcus strains, including *S. saprophyticus*. The higher lipolytic activity of *S. saprophyticus* DPC5671 compared with *C. casei* DPC5298 may explain the higher amount of FFAs in cheese A.

Numerous volatile compounds were significantly (P <0.05) associated to cheese A and B, especially those particularly characterised by strong aroma notes, such as some specific carboxylic acids, alcohols, esters, ketones and sulphur compounds, suggesting that the smear treatments have modified the aroma profile of Cheddar cheese curd in only 35 days of ripening (Table 2).

The metabolism of FAAs in cheese A and B in this study was responsible for the development of specific branched alcohols and branched chain acids detected (3-methyl-1-butanol, phenylethyl-alcohol, 3-methyl-butanoic acid). *D. hansenii* has been identified as a possible producer of alcohols in previous studies (Arfi, Spinnler, Tache, & Bonnarme, 2002; Gori, Sørensen, Petersen, Jespersen, & Arneborg, 2012; Leclercq-Perlat, Corrieu, & Spinnler, 2004b), while the production of carboxylic acids has been previously attributed to both yeasts and smear bacteria (*Geotrichum candidum* and *B. linens*) (Jollivet, Bézenger, Vayssier, & Belin, 1992; Jollivet, Chataud, Vayssier, Bensoussan, & Belin, 1994).

462 The high amount of esters detected in cheese A (ethyl acetate, ethyl octanoate, methyl
463 hexanoate, 3-methylbutyl acetate and isopentyl hexanoate) is likely related to the high FFA
464 content and the presence of alcohols in cheese A, considering they originate from the
465 esterification or alcoholysis of alcohols with carboxylic acids. While information on the
466 biosynthesis of esters by corynebacteria is sparse, numerous studies reported ester production
467 by staphylococci isolated from fermented foods, including *S. saprophyticus* strains (Talon,
468 Chastagnac, Vergnais, Montel, & Berdagué, 1998). The formation of esters in cheese A, not
469 detected in high amount in cheese B, is likely due to the metabolic activity of *S.*
470 *saprophyticus* DPC5671 rather than by *D. hansenii* DPC6258.
471 Other products of FFA metabolism such as ketones and alcohols were detected in all
472 cheeses. However 2-pentanone, 2-hexanone, 2-nonanone and 2-decanone were particularly
473 associated with the control cheese and not with cheese A and B, suggesting an involvement
474 of LAB rather than the smear cultures. It is known that methylketones result from the β-
475 oxidation of FFA, by lipolytic enzymes due to autolysis of the LAB during ripening (Collins,
476 McSweeney, & Wilkinson, 2003), although it is also postulated that methylketones can be
477 produced by the heating of milk or directly from esterification of β-keto acids (Alewijn,
478 2006; Forss, 1979).
479 Sensory analysis showed different results between cheese A, cheese B and the control
480 cheese. The sensory panel was not influenced by the colour of the cheese as the surface was
481 removed before sensory analysis. “Mould Cheese Flavour” and “Pungent Flavour” are
482 descriptors associated with smear-ripened cheeses and they can be correlated with a range of
483 volatiles with strong aroma notes (Table 2) detected on the surface and core of both cheese A
484 and B, namely butanoic, octanoic acid (originated from lipolysis of lipids), 2-heptanol (from
485 reduction of ketones), 2-methyl-propanoic, 3-methyl-butanoic, 2-methyl-butanoic, pentanoic
486 acid, 3-methyl-butanol, 2-methyl-butanol, phenylethyl-alcohol, 3-methyl-butanal (from

metabolism of branched chain amino acids or possibly phenylalanine for phenylethyl-alcohol), methanethiol, dimethyldisulphide and dimethyldisulphide (from metabolism of sulphur amino acids). As expected these descriptors were significantly associated (P <0.05) with cheese A and/or B, suggesting that the activities of yeast and Gram-positive bacteria on the cheese conferred a typical smear cheese flavour, not perceived in the control. The descriptors significantly associated (P <0.05) with cheese B, such as "Sweaty/Sour Aroma", "Pungent Aroma" and "Off-Aroma", are considered as "unclean" and off-odorants and were associated with some compounds detected in abundance on the surface and core of cheese B, such as 3-methyl-butanoic acid, octanoic acid, methanethiol and particularly 8-nonen-2-one (originated from β-oxidation of fatty acids) which was identified only in cheese B.

Sensory analysis showed the smear cultures on the cheese surface affected the cheese ripening giving strong and intense aroma and flavour to cheese A and B, while the control cheese was characterised by mild aroma and flavour. In a short ripening time of 35 days, the smear treatments induced the development of different aroma profiles.

Overall, the bacterial strains in conjunction with *D. hansenii* may have the potential to modify cheese colour and produce novel cheeses with diverse aromas using a Cheddar cheese curd.

5. Conclusion

The cheese-making method described in this paper gives a new approach for the production of novel smear cheeses starting from a Cheddar cheese curd. Both the yeast and bacterial cultures were able to establish themselves on the surface of the cheese and become the dominant microbiota on the cheese surface, producing a cheese variety with acceptable appearance and novel flavour aroma profiles. The method proposed could be used as model

to produce novel cheese types with a range of flavours and aromas through the growth of combinations of yeast and bacterial cultures on the surface using cheese curd produced on a traditional Cheddar cheese plant.

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Figure legends

Fig. 1. Enumeration of cfu g^{-1} of the cheese surface of cheese A and cheese B during the ripening time; yeasts total count in cheese A (●) and in cheese B (⊗), smear bacteria total count in cheese A (△) and in cheese B (□). Values presented are the means and standard deviations from three replicate trials.

Fig. 2. Change in pH overtime; control cheese (●), surface of cheese A (△), core of cheese A (▲), surface of cheese B (□) and core of cheese B (■). Values presented are the means and standard deviations from three replicate trials.

Fig. 3. Urea-PAGE electrophoretogram of sodium caseinate (lane C), control cheese at day 0 (lane 1), control cheese at day 35 (lane 2), core of cheese B at day 35 (lane 3), surface of cheese B at day 35 (lane 4), core of cheese A at day 35 (lane 5) and surface of cheese A at day 35 (lane 6). The urea-PAGE was performed on the basis of fixed weight protein for all three replicate trials.

Fig. 4. Individual free amino acids content of (A) the control (■), core cheese A (▩), surface cheese A (▤), and (B) the control (■), core cheese B (▩) and surface cheese B (▤) at day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different (P <0.05).

Fig. 5. Individual free fatty acids content of (A) the control (■), core cheese A (▩) and surface cheese A (▤), and (B) the control (■), core cheese B (▩) and surface cheese B (▤) at

day 35. The values presented are the means of the three replicate trials; values within an amino acid group with different letters are significantly different (P <0.05).

Fig. 6. Colour development on the surface of the cheeses during the ripening. The colour values (L^*, a^*, b^*) of the control cheese (●), of cheese A (▲) and of cheese B (□). Values presented are the means and standard deviations from three replicate trials.

Fig. 7. Sensory affective (hedonic) analysis performed in duplicate on cheese A (▲), cheese B (■) and control (●) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher (P <0.05) than cheese A and B; (e) cheese A and control significantly higher (P <0.05) than cheese B.

Fig. 8. Ranking descriptive analysis performed in duplicate on cheese A (▲), cheese B (■) and control (●) at 35 days of ripening. Letters after an attribute descriptor denote: (a) control significantly higher (P <0.05) than cheese A and B; (b) cheese A and B significantly higher (P <0.05) than control; (c) cheese B significantly higher (P <0.05) than cheese A and control; (d) cheese A significantly higher (P <0.05) than cheese B and control.

Table 1

Composition of the control at day 0 and 35 and composition of the surface and core of cheese A and cheese B at day 35. [a]

Composition	Control d0	Control d35	Surf A d35	Core A d35	Surf B d35	Core B d35
Moisture (%, w/w)	38.80±0.84	38.32±0.62[c]	39.71±1.20	40.49±0.86	39.61±1.43	41.27±0.39[a]
Fat (%, w/w)	30.45±0.33	30.41±0.27	28.30±0.14[b]	30.09±0.69	28.01±0.25[b]	29.80±0.30
Protein (%, w/w)	24.87±0.21	25.17±0.11	24.28±0.57	24.5±0.83	24.94±0.32	24.72±0.44
MNFS (%, w/w)	55.79±1.44	55.07±1.02[c]	55.39±1.77	57.89±1.33	55.02±2.13[b]	58.79±0.39[a]
FDM (%, w/w)	49.77±1.16	49.32±0.80	46.97±1.13[b]	50.51±1.33	46.41±1.44[b]	50.74±0.24
S/M (%, w/w)	4.02±0.10	4.17±0.26[a]	3.74±0.20	3.81±0.33	3.58±0.10[b]	3.88±0.51
Salt (%, w/w)	1.56±0.02	1.60±0.09	1.48±0.11	1.55±0.12	1.42±0.08	1.60±0.22

[a] The compositional values are for moisture, fat, protein, MNFS (moisture in non-fat substances), FDM (fat in dry matter), S/M (salt in moisture) and salt of control at day 0 (Control d0), for control at day 35 (Control d35), for the surface of cheese A at day 35 (Surf A d35), for the core of cheese A at day 35 (Core A d35), for the surface of cheese B at day 35 (Surf B d35) and for the core of cheese B at day 35 (Core B d35). Values presented are the means±standard deviations of three replicate trials; values with different superscript letters differ significantly (P <0.05).

Table 2

Volatile compounds detected with SPME-GCMS in cheese A, cheese B and control, and relative aroma notes. [a]

Volatile compound	CAS number	Aroma note
Aldehydes		
3-Methyl-butanal[b]	590-86-3	Malty, powerful, cheese, green, dark chocolate (Kilcawley, 2017)
2-Methyl butanal[b]	96-17-3	Malty, dark chocolate, almond, cocoa (Qian et al., 2006; Singh et al., 2003; Urbach, 1993)
Alcohols		
Ethanol[e]	64-17-5	Dry, dust, alcohol (Kilcawley, 2017)
2-Butanol[e]	78-92-2	Sweet, fruity, fusel oil, wine-like (Kilcawley, 2017)
3-Methyl-1-butanol[a]	123-51-3	Fresh cheese, breath-taking, alcoholic, fruity, grainy (Kilcawley, 2017)
2-Methyl-1-butanol[a]	137-32-6	Malty, wine, onion (Kilcawley, 2017)
2,3-Butanediol[e]	513-85-9	Fruity (Singh et al., 2003)
2-Heptanol[a,b]	543-49-7	Fruity, earthy, green, sweetish, dry (Kilcawley, 2017)
2-Ethyl-1-hexanol[d,e]	104-76-7	Animal, cardboard (Thomsen et al., 2012)
Phenylethyl-alcohol[a]	60-12-8	Unclean, rose, violet-like, honey, floral (Kilcawley, 2017)
Ketones		
2,3-Butanedione[c]	431-03-8	Buttery, strong (Kubíčková & Grosch, 1997; Singh et al., 2003)
2-Pentanone[e]	107-87-9	Orange peel, sweet, fruity (Kilcawley, 2017)
3-Methyl-2-pentanone[a]	565-61-7	Minty-camphoraceous, sharp (Barron et al., 2005)
2-Hexanone[e]	591-78-6	Floral, fruity (Qian et al., 2006)
8-Nonen-2-one[d]	5009-32-5	Animal, stinky (Poveda et al., 2008; Varming et al., 2013)
2-Nonanone[e]	821-55-6	Malty, rotten fruit, hot milk, green, earthy (Kilcawley, 2017)
2-Decanone[e]	693-54-9	Fruity, musty (Qian et al., 2006; Varming, et al., 2013)
Acids		
Acetic acid[a]	64-19-7	Vinegar, peppers, green, fruity, floral (Kilcawley, 2017)
2-Methyl-propanoic acid[a]	79-31-2	Rancid butter, sweaty, sweet, apple-like (Curioni & Bosset, 2002)
Butanoic acid[a]	107-92-6	Sweaty, butter, cheese, strong, acid (Kilcawley, 2017)
3-Methyl-butanoic acid[b]	503-74-2	Cheesy, sweaty, socks, rancid, rotten fruit (Kilcawley, 2017)
2-Methyl-butanoic acid[a]	116-53-0	Fruity, waxy, sweaty (Singh et al., 2003)
Pentanoic acid[a]	109-52-4	Rain, wood, vegetable, spicy, nutty, grain, Swiss cheese, stable, sweaty, sheep (Curioni & Bosset, 2002)
Heptanoic acid[a]	111-14-8	Soapy, fatty, goaty, rancid (Curioni & Bosset, 2002)
Octanoic acid[b]	124-07-2	Cheesy, rancid, pungent, sweat (Kilcawley, 2017)
n-Decanoic acid[a]	334-48-5	Stale, butter, sour, fruity, pungent (Kilcawley, 2017)
Esters		
Ethyl acetate[a]	141-78-6	Solvent, pineapple, fruity (Kilcawley, 2017)
Ethyl propionate[e]	105-37-3	Pineapple, solvent, fruity (Barron et al., 2005; Qian et al., 2006)
3-methylbutyl acetate[a]	123-92-2	Fruity, banana, candy, sweet (Barron et al., 2005; Curioni & Bosset, 2002; Qian et al., 2006)
Methyl hexanoate[a]	106-70-7	Pineapple, fruity (Qian et al., 2006; Varming et al., 2013)
Ethyl hexanoate[b]	123-66-0	Pineapple, sweet, fruity, banana (Kilcawley, 2017)
Ethyl octanoate[a]	106-32-1	Pear, apricot, sweet, fruity, banana, pineapple (Kilcawley, 2017)
Isopentyl hexanoate[a]	2198-61-0	Sweet, fruity (Gürbüz et al., 2006)
Sulphur compounds		
Methionol[a]	505-10-2	Orange (Carpino et al., 2004)
Methanethiol[a,b]	74-93-1	Rotten cabbage, cheese, vegetative, sulphur (Kilcawley, 2017)
Dimethyldisulphide[a]	624-92-0	Green, sour, onion (Kilcawley, 2017)
Dimethyltrisulphide[a]	3658-80-8	Vegetable-like, sulphurous, garlic, putrid, cabbage-like (Kilcawley, 2017)
Aromatic hydrocarbons		
Benzaldehyde[c]	100-52-7	Bitter almond, sweet cherry (Singh et al., 2003; Smit et al., 2005)
Benzeneacetaldehyde[a]	122-78-1	Honey-like, rose, violet-like, hyacinth, green (Kubíčková & Grosch, 1997; Qian et al., 2006; Singh et al., 2003; Smi, et al., 2005; Varming et al., 2013)
Pyrazines		
3-Ethyl-2,5-dimethyl-pyrazine[b]	13360-65-1	Roasted, baked (Qian & Reineccius, 2002)

[a] Superscript letters denote that volatile compounds are significantly different (P<0.05) and positively correlated to: ([a]) the surface of cheese A; ([b]) the surface of cheese B; ([c]) the core of cheese A; ([d]) the core of cheese B; ([e]) the control cheese.

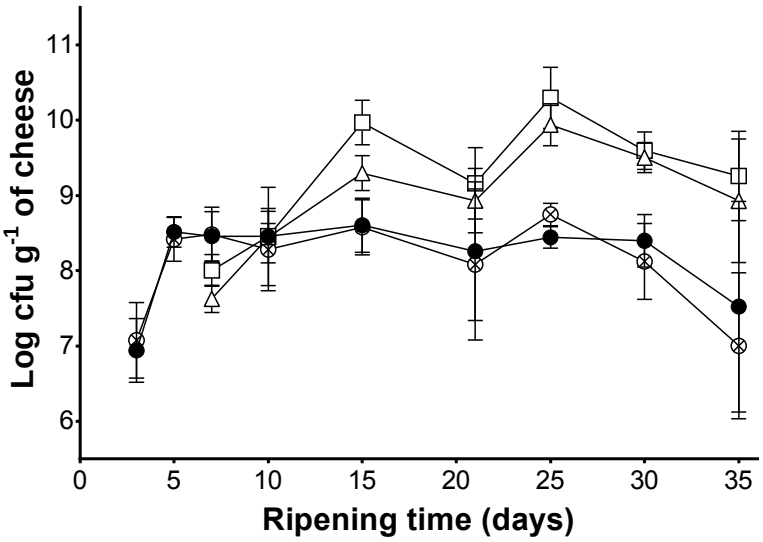

Fig. 1.

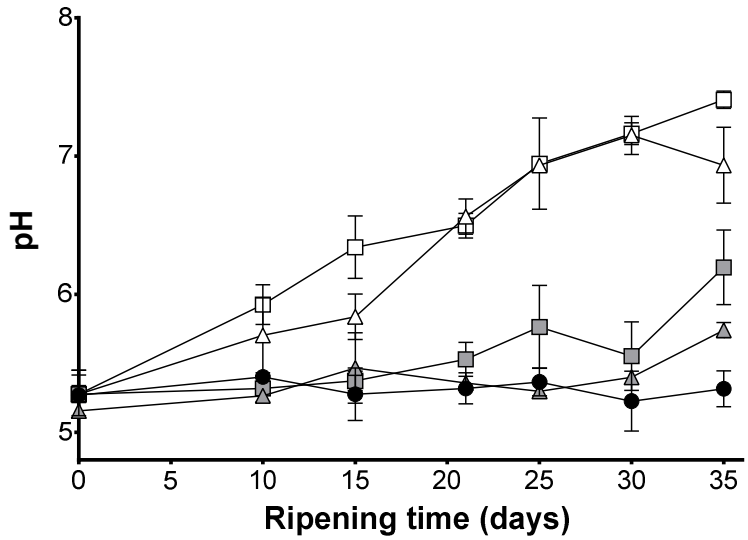

Fig. 2.

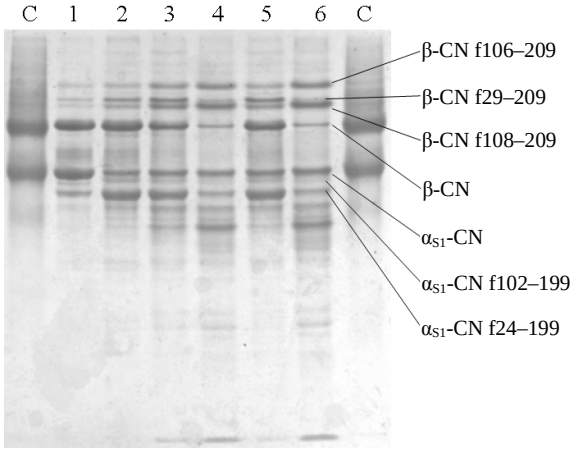

Fig. 3.

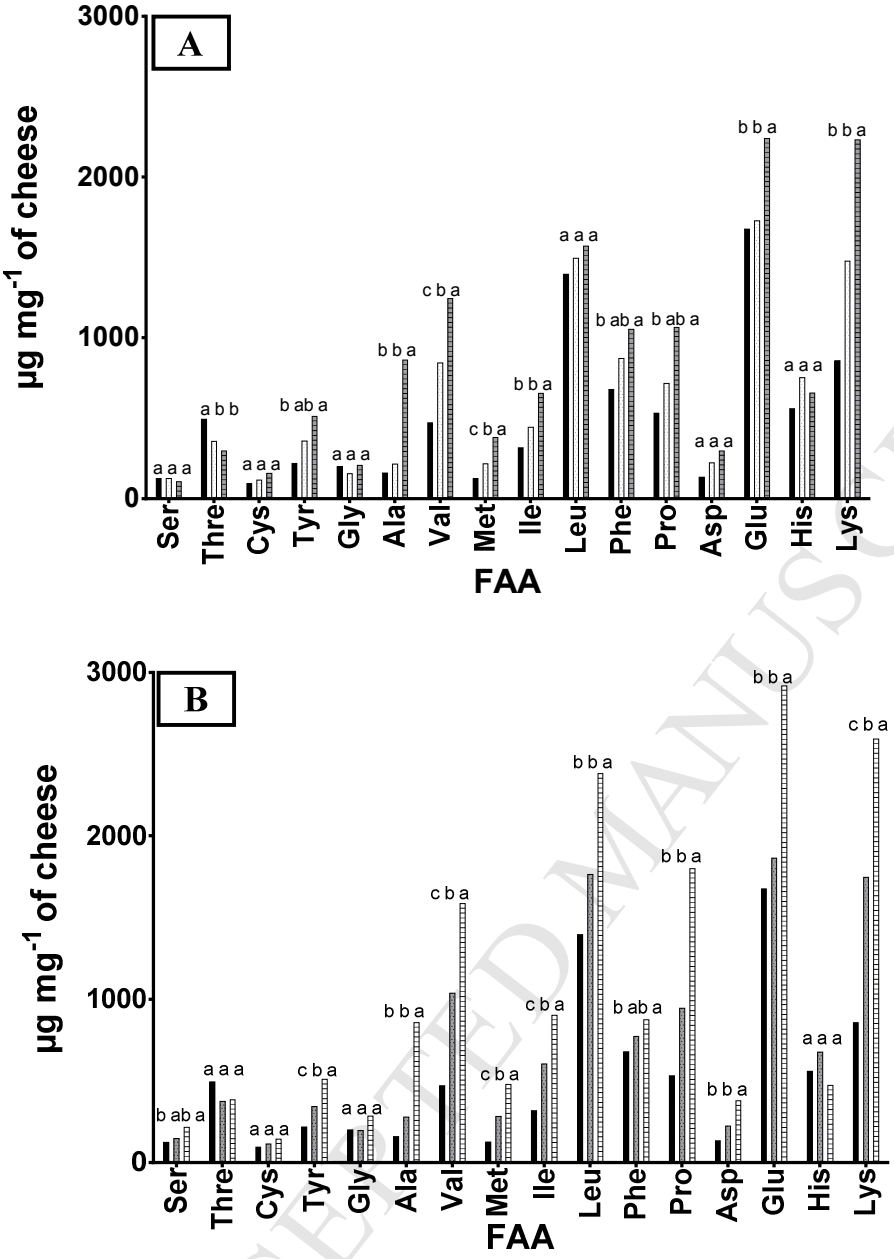

Fig. 4.

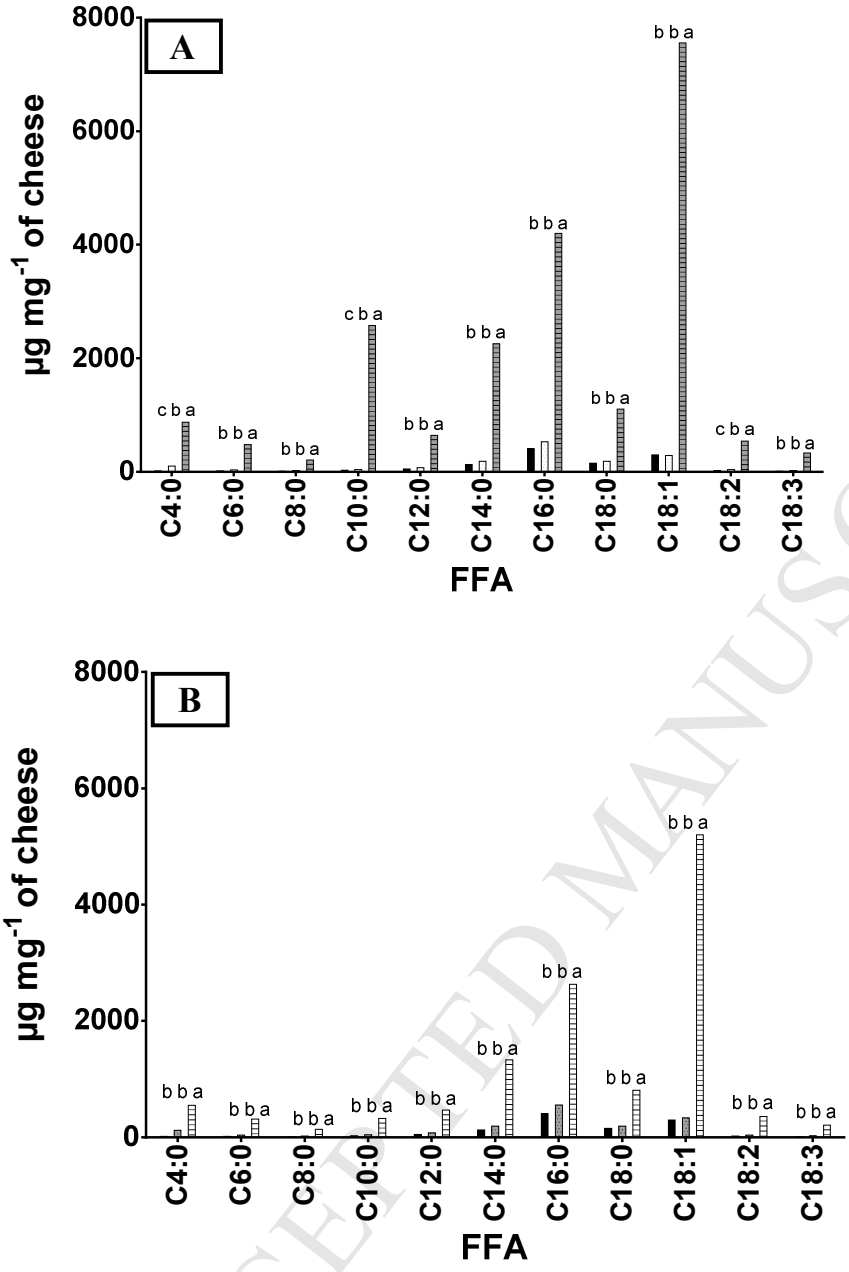

Fig. 5.

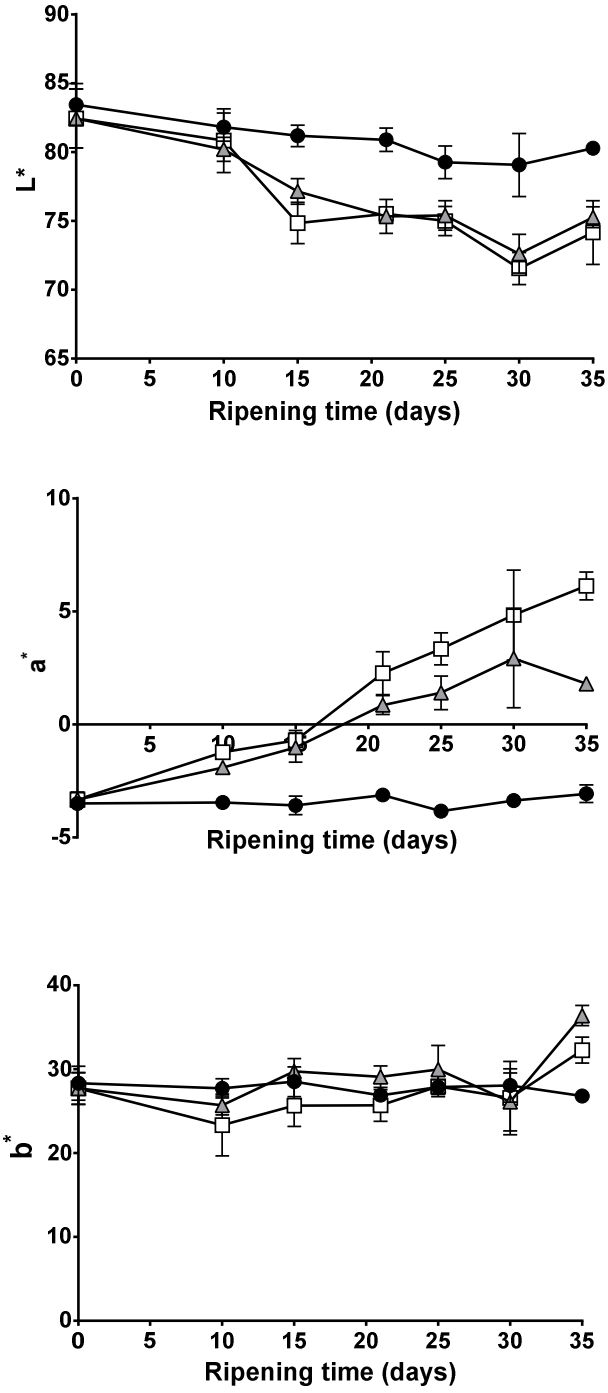
L*
Ripening time (days)
a*
Ripening time (days)
b*
Ripening time (days)

Fig. 6.

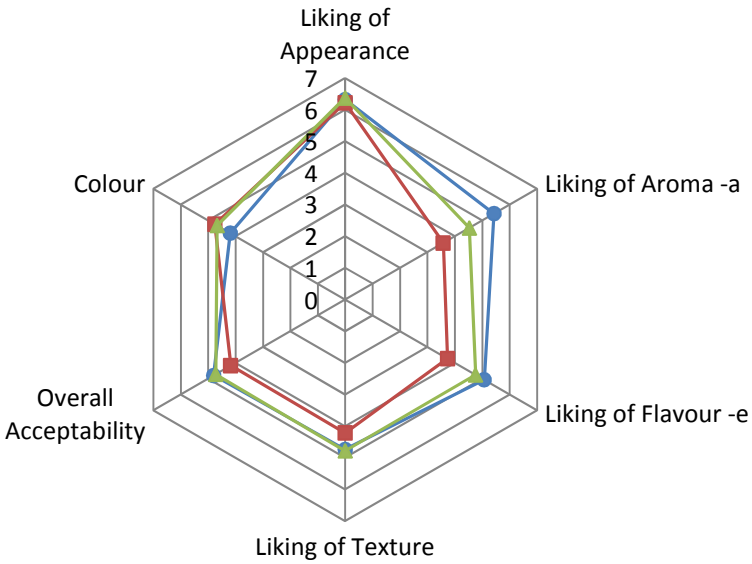

Fig. 7.

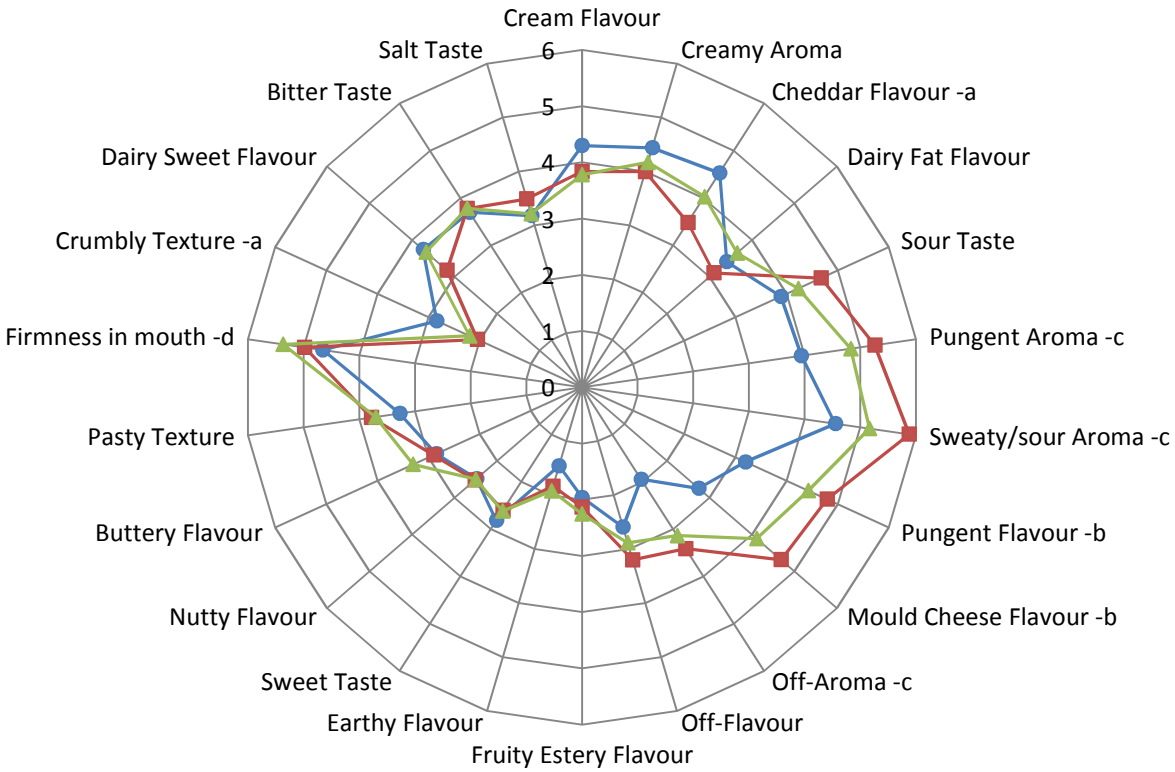

Fig. 8.